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Exhibit A

Structural and functional features of a specific nucleosome containing a recognition element for the thyroid hormone receptor

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The *Xenopus* thyroid hormone receptor βA (TRβA) gene contains an important thyroid hormone response element (TRE) that is assembled into a positioned nucleosome. We determine the translational position of the nucleosome containing the TRE and the rotational positioning of the double helix with respect to the histone surface. Histone H1 is incorporated into the nucleosome leading to an asymmetric protection to micrococcal nuclease cleavage of linker DNA relative to the nucleosome core. Histone H1 association is without significant consequence for the binding of the heterodimer of thyroid hormone receptor and 9-*cis* retinoic acid receptor (TR/RXR) to nucleosomal DNA *in vitro*, or for the regulation of TRβA gene transcription following microinjection into the oocyte nucleus. Small alterations of 3 and 6 bp in the translational positioning of the TRE in chromatin are also without effect on the transcriptional activity of the TRβA gene, whereas a small change in the rotational position of the TRE (3 bp) relative to the histone surface significantly reduces the binding of TR/RXR to the nucleosome and decreases transcriptional activation directed by TR/RXR. Our results indicate that the specific architecture of the nucleosome containing the TRE may have regulatory significance for expression of the TRβA gene.

Keywords: chromatin structure/linker histones/nuclear receptors/nucleosome positioning/transcriptional regulation

Introduction

The assembly of specific regulatory nucleoprotein architectures is a common feature of many eukaryotic promoters (Becker, 1994; Wallrath *et al.*, 1994; Wolffe, 1994). Within these architectures, histone–DNA contacts may be precisely arranged so as to allow access of transcription factors to nucleosomal DNA (Perlmann and Wrange, 1988; Schild *et al.*, 1993; Truss *et al.*, 1995). Nucleosome positioning is a common organizational feature within the chromatin of eukaryotic promoters (Simpson, 1991; Thoma, 1992; Wolffe, 1994). The stability of positioned nucleosomes (Straka and Horz, 1991) and their exact placement (Wolffe and Drew, 1989; Simpson, 1990) can influence the function of regulatory DNA *in vivo* and *in vitro*. We have been interested in the role of nucleosome positioning in the regulation of the *Xenopus* TRβA gene by the thyroid hormone receptor (Wong *et al.*, 1995, 1997). A TRE within the TRβA gene is rotationally positioned on the surface of the nucleosome such that the TR/RXR can still bind in chromatin *in vivo* and *in vitro* (Wong *et al.*, 1995). The capacity of the TR/RXR to bind stably in chromatin may have an important role both in potentially targeting histone deacetylase for transcriptional silencing (Heinzel *et al.*, 1997; Nagy *et al.*, 1997) and in targeting chromatin disruption on addition of thyroid hormone (Wong *et al.*, 1995, 1997; Ogrzyko *et al.*, 1996; Yang *et al.*, 1996).

Various structural and sequence features of DNA contribute to nucleosome positioning through their influence on the wrapping of DNA around the histone octamer. These include the anisotropic flexibility of the double helix (Shrader and Crothers, 1989), intrinsic DNA curvature (Wolffe and Drew, 1989), inflexible DNA sequences (Prunell, 1982) and CTG triplet repeats (Godde and Wolffe, 1996). DNA curvature is one of the best understood determinants of the rotational orientation of the double helix relative to the surface of the core histones (Drew and Travers, 1985). Narrow minor grooves face in towards the histones, and wide minor grooves face out towards solution. Although some of the determinants of the rotational positioning of DNA on the surface of the histone octamer are reasonably well understood (Calladine and Drew, 1986), this is not true for translational positioning of the histones with respect to DNA sequence. The translational positioning of the histone octamer along the DNA sequence is much more variable than the rotational positioning of DNA in the nucleosome (Dong *et al.*, 1989; Meersseman *et al.*, 1991; Ura *et al.*, 1995). Several translational positions that differ from each other by integral turns of the double helix are often observed (Ura *et al.*, 1995). This probably reflects the similarity in binding energy of the histone octamer to DNA sequences that have a similar curvature or anisotropic flexibility and that differ only in where exactly on a DNA curve the first contact with the core histones is made (Hayes *et al.*, 1991). Histone H1 has the capacity to influence the exact position of the histone octamer with respect to DNA sequence (Meersseman *et al.*, 1991; Ura *et al.*, 1995). This influence may occur as a consequence of the winged-helix domain of histone H1 binding to DNA in the nucleosome and making sequence-selective contacts with the DNA major groove in a comparable way to the transcription factor HNF-3 (Clark *et al.*, 1993; Ramakrishnan *et al.*, 1993; Pruss *et al.*, 1996).

Here we examine some of the structural features of the specific nucleosome that includes the thyroid hormone response element (TRE) within the *Xenopus* TRβA gene. We find that the core histones alone show strong selectivity for translational positioning along this DNA sequence. Incorporation of histone H1 preferentially extends histone–DNA interactions to one side of the nucleosome core, but does not influence the rotational positioning of DNA on the histone surface. Nor does histone H1 influence the association of TR/RXR with the TRE within the positioned nucleosome. Alterations in the rotational positioning of the TRE on the surface of the histone octamer are difficult to achieve unless strong nucleosome positioning sequences from the *Xenopus borealis* 5S rRNA gene (Hayes *et al.*, 1990; Godde *et al.*, 1995) are used to override those from the TRβA gene. Under these circumstances small perturbations in the rotational position of the TRE are found to influence TR/RXR binding. Consistent with our *in vitro* binding studies, we find that expression of histone H1 is without effect on the transcription of the TRβA promoter *in vivo*, but that alterations in the rotational positioning of the TRE in the nucleosome can influence transcriptional efficiency.

Results

Nucleosome reconstitution on the TRβA gene

Our first experiments were to reconstitute mononucleosomes and dinucleosomes on DNA fragments containing TRβA gene sequences. We made use of a 204 bp fragment from +159 to +363 relative to the start site of transcription (+1) for mononucleosome assembly and a 377 bp fragment from +159 to +536 for dinucleosome assembly. The choice of these DNA sequences was based on low-resolution mapping of nucleosome positions within minichromosomes assembled on the TRβA gene sequences following microinjection into the *Xenopus* oocyte nucleus (data not shown; Wong *et al.*, 1995). The reconstituted chromatin was fractionated on a sucrose gradient and each fraction was analyzed by nucleoprotein gel electrophoresis (Figure 1A). Free DNA was resolved from mono- and dinucleosomal complexes. Mono- and dinucleosomal complexes were used for the reconstitution of linker histones. Titration with increasing amounts of histone H1 led to a supershift of the mononucleosome (Figure 1B) and dinucleosome complex (Figure 1C). An additional assay for the stable inclusion of linker histones into chromatin is the appearance of DNA fragments of distinct sizes following micrococcal nuclease digestion (Simpson, 1978; Allan *et al.*, 1980; Hayes and Wolffe, 1993). Core particles containing 146 bp of DNA accumulate during micrococcal nuclease digestion in the presence of histone octamers alone, using both mononucleosome (Figure 1D, lanes 3 and 4) and dinucleosome templates (Figure 1E, lanes 4 and 5). In the presence of histone octamers and linker histones, chromatosome particles accumulate during digestion containing all of the histones and >166 bp of DNA (Figure 1D, lane 1 and Figure 1E, lane 2). We conclude that linker histones are stably incorporated into the mono- and dinucleosomal templates under our reconstitution conditions (see also Hayes and Wolffe, 1993; Ura *et al.*, 1995).

Translational positioning of nucleosomes on the TRβA

In earlier work we have suggested that the core histones occupy non-random positions with respect to the DNA sequence of the TRβA gene (Wong *et al.*, 1995). We investigated this issue further using a nuclease mapping methodology that can determine the exact DNA sequences where histone–DNA contacts start and finish in a nucleosome core or in a chromatosome (Dong *et al.*, 1989; Meersseman *et al.*, 1991). Our first analysis made use of nucleosome cores assembled using mononucleosome templates. Micrococcal nuclease digestion was followed by restriction endonuclease mapping of the translational positions of the histone octamer relative to the TRβA gene sequence (Figure 2A, lanes 1–5). We find that the 5′ boundary of the histone octamer has a major position at +205 (Figure 2A, lanes 3 and 4, upper dots). There are two other minor 5′ boundaries spaced at 10–11 bp from this major position towards the 5′ end of the DNA sequence. We next examined the boundaries of the chromatosome fragments reconstituted using the mononucleosome template (Figure 2A, lanes 6–10). The 5′ boundary of the histone–DNA complex becomes more restricted in the distribution of DNA fragment lengths, and moves 20 bp towards the 5′ end of the DNA sequence (Figure 2A, lanes 8 and 9, upper dots). In contrast, the 3′ boundaries remain the same for both core and chromatosome particles (Figure 2A, lanes 3, 4, 8 and 9, lower dots). Thus inclusion of histone H1 into the nucleosome extends histone–DNA contacts asymmetrically relative to the boundaries of the nucleosome core on a defined DNA sequence (see also Hayes and Wolffe, 1993; Ura *et al.*, 1995; Nightingale *et al.*, 1996b).

We extended our analysis to the dinucleosomal complex. Our mapping of the 5′ nucleosome boundaries in the presence or absence of histone H1 was identical to those of the mononucleosome (data not shown). For the 3′ nucleosome, both nucleosome cores and chromatosomes had a high level of translational positioning with respect to DNA sequence (Figure 2B, lanes 3 and 8). Note that in this protocol we predigested the DNA with enzymes unique to the 5′ nucleosome to eliminate their input to the mapping of the 3′ nucleosome boundaries. Addition of histone H1 generated a highly asymmetric protection of DNA sequence relative to the nucleosome core boundaries (Figure 2B, compare lanes 3 and 8). We conclude that the TRβA DNA sequences downstream from the start site of transcription have the capacity to direct the translational positioning of histone octamers. We also note that inclusion of histone H1 does not alter the DNA sequences occupied by the nucleosome core, but extends the region of DNA protected by the nucleosome by 20 bp in a highly asymmetric position.

Rotational positioning of the TRβA gene sequence in the nucleosome

We next examined the detailed rotational positioning of the TRβA gene sequences from +205 to +351 on the surface of the histones in the presence or absence of histone H1. DNase I footprinting of this DNA sequence in association with the core histones has previously been shown to give a highly periodic cleavage pattern, reflecting

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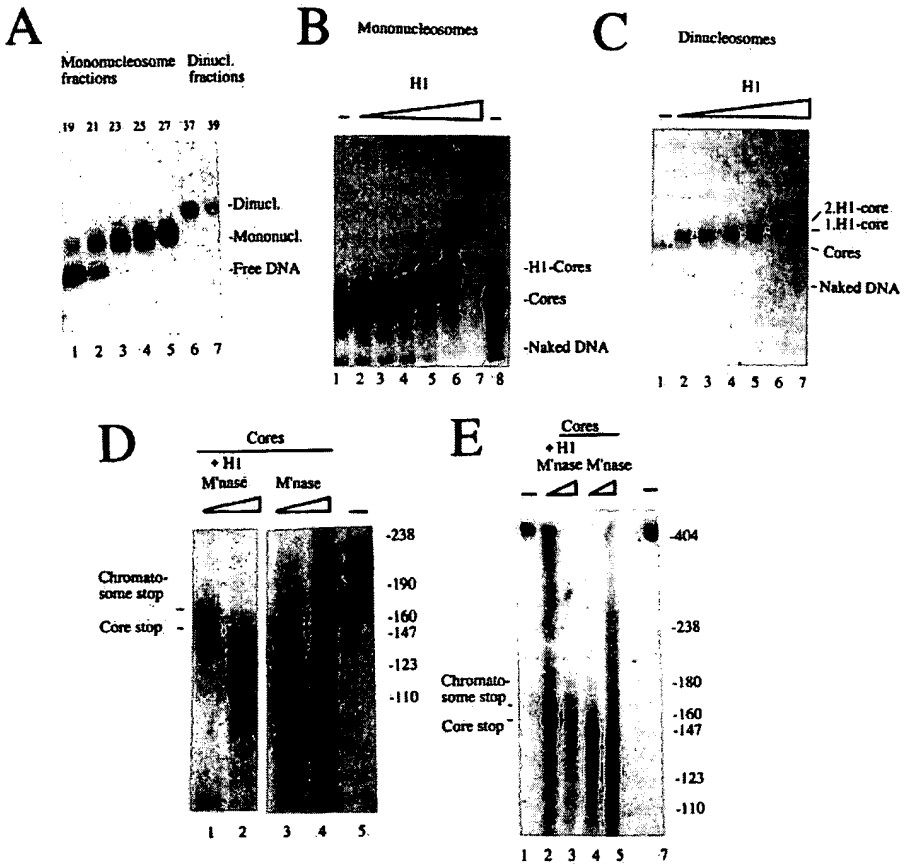

Fig. 1. Preparation and characterization of reconstituted TRβA mononucleosomes and dinucleosomes. (**A**) Radiolabeled DNA templates, for mononucleosomes a 240 bp fragment (from +159 to +363) and for dinucleosomes a 377 bp fragment (from +159 to +536) were reconstituted at a molar ratio of core histones to DNA of 1.0 with purified core histone proteins. The products were fractionated on 5–20% sucrose gradients and each fraction was analyzed by nucleoprotein gel electrophoresis. The numbers above the gel lanes refer to individual fractions, the right-hand fractions are towards the bottom of the gradient. The mononucleosomes from one gradient are shown in fractions 19 to 27 (lanes 1–5), and the dinucleosomes from a separate gradient in fractions 37 and 39 (lanes 6 and 7) are shown. The positions of free DNA, mononucleosomes and dinucleosomes are shown. (**B**) Binding of histone H1 to reconstituted mononucleosomes. Reconstituted mononucleosome cores were mixed with various amounts of histone H1 and analyzed on a 4% non-denaturing acrylamide gel (see Materials and methods). Lanes 1–8, 100 ng (DNA content) of reconstituted nucleosome cores (= 1.2 pmol) was mixed with zero (lanes 1 and 8), 0.3, 0.6, 0.9, 1.2, 1.8 and 2.4 pmol of H1 respectively (lanes 2–7). The positions of naked DNA, nucleosome cores (Cores) and the nucleosomes containing histone H1 (H1-Cores) are indicated. (**C**) Binding of histone H1 to reconstituted dinucleosomes. Reconstituted dinucleosome cores were mixed with various amounts of histone H1 and analyzed by non-denaturing agarose (0.7%) gel electrophoresis (see Materials and methods). Lanes 1–7, 100 ng (DNA content of reconstituted dinucleosome cores (= 0.6 pmol) was mixed with 0, 0.3, 0.6, 0.9, 1.2, 1.5 and 1.8 pmol of histone H1 respectively (lanes 1–7). The positions of naked DNA, dinucleosome cores (Cores), dinucleosome cores with a single molecule of H1 (1H1-Cores) and dinucleosome cores with two molecules of H1 (2H1-Cores) are indicated. The dot between lanes 1 and 2 indicates the position of the dinucleosome cores; that between lanes 2 and 3, the position of the dinucleosome cores with a single molecule of H1; and that between lanes 5 and 6, dinucleosome cores with two molecules of H1. A short exposure of this gel is shown to facilitate resolution of the different nucleoprotein complexes. (**D**) Micrococcal nuclease digestion of the reconstituted mononucleosome. Reconstituted mononucleosome (80 ng of DNA, lanes 1–5) in the presence (lanes 1 and 2) or absence (lanes 3 and 4) of 16 ng histone H1 (moles of histone H1/mononucleosome = 1) were digested with 0.15 and 0.6 U of micrococcal nuclease (5 min, 22°C) as indicated by the triangles above the lanes. No enzyme was added to the sample shown in lane 5. Products of digestion were labeled with [γ-^{32}P]ATP and analyzed by native polyacrylamide (6%) gel electrophoresis. Markers are end-labeled *Msp*I-digested pBR322 size markers. Arrows indicate the core particle (core stop) and chromatosome (chromatosome stop) products of digestion respectively. (**E**) As in (D) except dinucleosomes were used. Reconstituted dinucleosomes (80 ng of DNA, lanes 1–7) in the presence (lanes 2 and 3) or absence (lanes 4 and 5) of 16 ng histone H1 (moles of H1/dinucleosome = 2) were digested with 0.15 and 0.6 U of micrococcal nuclease (5 min, 22°C) as indicated by the triangles above the lanes. No enzyme was added to the samples shown in lanes 1 and 7.

a precise rotational positioning of the double helix on the histone surface (Wong *et al.*, 1995). Hydroxyl radical cleavage is a favored reagent for examining the rotational positioning of DNA in the nucleosome due to the small size of the hydroxyl radical and lack of sequence preference in DNA cleavage (Hayes *et al.*, 1990, 1991). Hydroxyl radical cleavage of the TRβA nucleosome reveals strong rotational positioning of the double helix on the surface of the histone octamer (Figure 3A, lanes 2–4). This positioning is unchanged on incorporation of histone H1 into the nucleosome (Figure 3A, compare lanes 3 and 4). The hydroxyl radical cleavage patterns (Figure 3A), together with the translational mapping of the histone octamer with respect to DNA sequence (Figure 2) allows an approximate model of the TRβA gene nucleosome to be generated (Figure 3C). In this model the TRE (hatched DNA sequence) lies across the nucleosome dyad. The boxed sequences show the key base pairs recognized by

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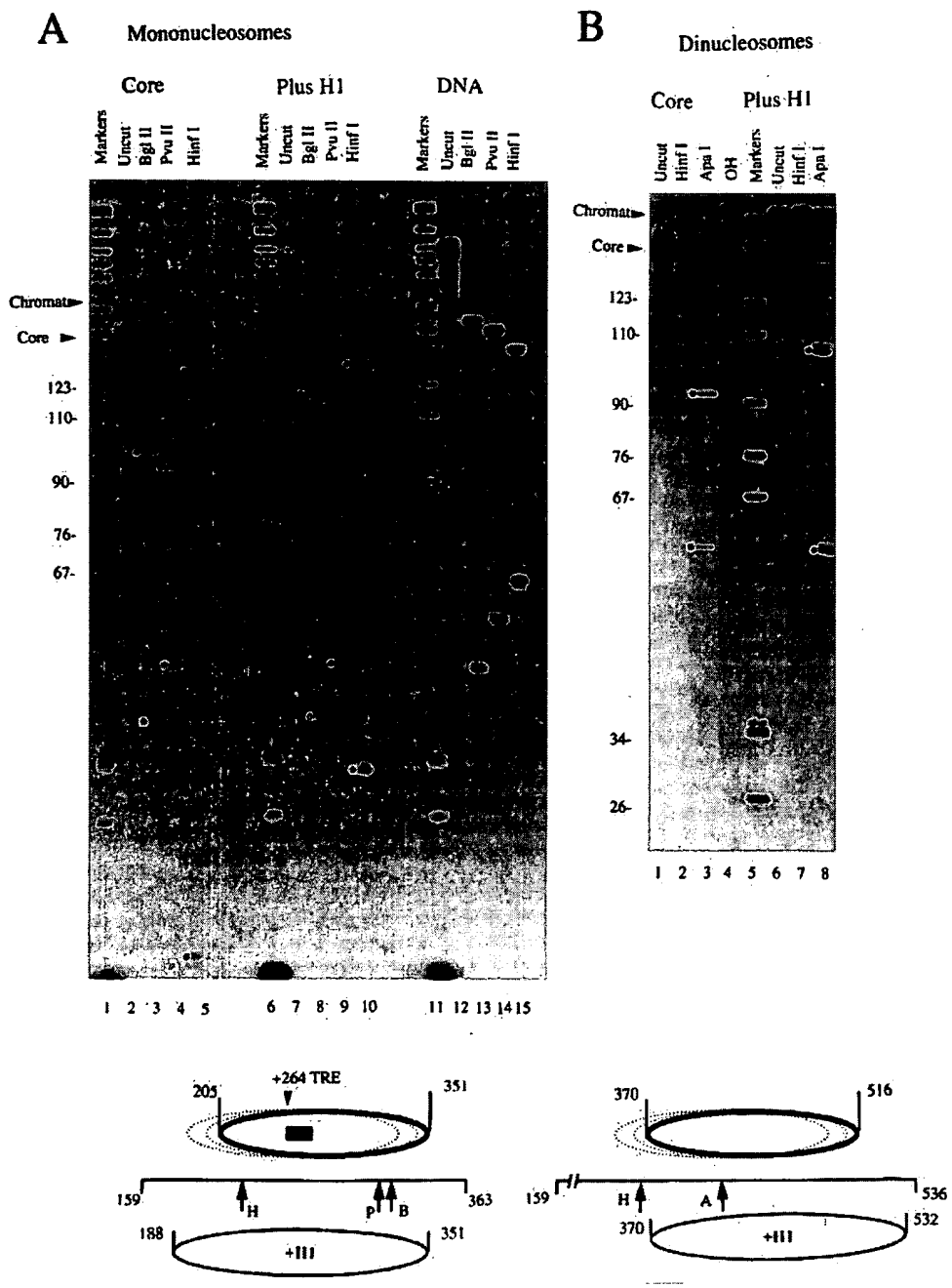

Fig. 2. Micrococcal nuclease mapping of core and chromatosome positions on reconstituted mononucleosome and dinucleosome complexes. DNA from the nucleosome core particle and the chromatosome was recovered from a non-denaturing acrylamide gel (see Figure 1D and E) and digested with restriction enzymes to determine the positions of the boundaries of histone–DNA complexes. In the case of the mapping of the 3′ nucleosome of the dinucleosome template, DNA was predigested with restriction enzymes *Pvu*II and *Bgl*II, which are specific for the 5′ nucleosome in order to eliminate contributions from this nucleosome in the boundary analysis. (**A**) Mapping the boundaries of the mononucleosome (the 5′ nucleosome of the dinucleosome reconstitute). Lanes 1–5, mapping of core particle boundaries. Lane 1 contains *Msp*I-digested pBR322 size markers. Lane 2, uncut core particle DNA (146 bp in length). Lane 3, core particle DNA digested with *Bgl*II. The dots indicate the predominant DNA fragments that together generate 146 bp. Lane 4, core particle DNA digested with *Pvu*II. Lane 5, core particle DNA digested with *Hin*fI. Lanes 6–10, as in lanes 1–5 except that chromatosome length DNA (~166 bp) is used. The dots indicate predominant DNA fragment lengths that together generate ~166 bp. Lanes 11–15, as in lanes 1–5 except intact DNA used for reconstitution is used. A scheme is shown in the lower panel of the boundaries of the nucleosome core (bold ellipsoid) and the approximate position of the TRE (black bar). Minor translational positions are shown as dotted ellipsoids. The sites of cleavage by the restriction endonucleases *Hin*fI (H), *Pvu*II (P) and *Bgl*II (B) are indicated. The lowest ellipsoid indicates the boundaries of the chromatosome in the presence of histone H1. (**B**) Mapping of the boundaries of the 3′ nucleosome of the dinucleosome reconstitute. Lanes 1–3 show mapping of core particle boundaries. Lane 1 is uncut core particle DNA (146 bp in length). Lane 2 shows cleavage (or more accurately lack of cleavage) by *Hin*fI; lane 3 shows cleavage by *Apa*I. The dots indicate DNA fragments whose length together generates 146 bp. Lane 4 shows a hydroxyl radical-cleaved, end-labeled probe which provides markers. Lane 5 contains *Msp*I-digested pBR322 size markers. Lanes 6–8 are as in lanes 1–3 except that chromatosome length DNA (~166 bp) is used. The dots indicate predominant DNA fragment lengths that together generate ~166 bp. A scheme is shown in the lower panel of the boundaries of the nucleosome core (bold ellipsoids). The sites of cleavage by the restriction endonucleases *Hin*fI (H) and *Apa*I (A) are indicated. The lowest ellipsoid indicates the boundaries of the chromatosome in the presence of histone H1.

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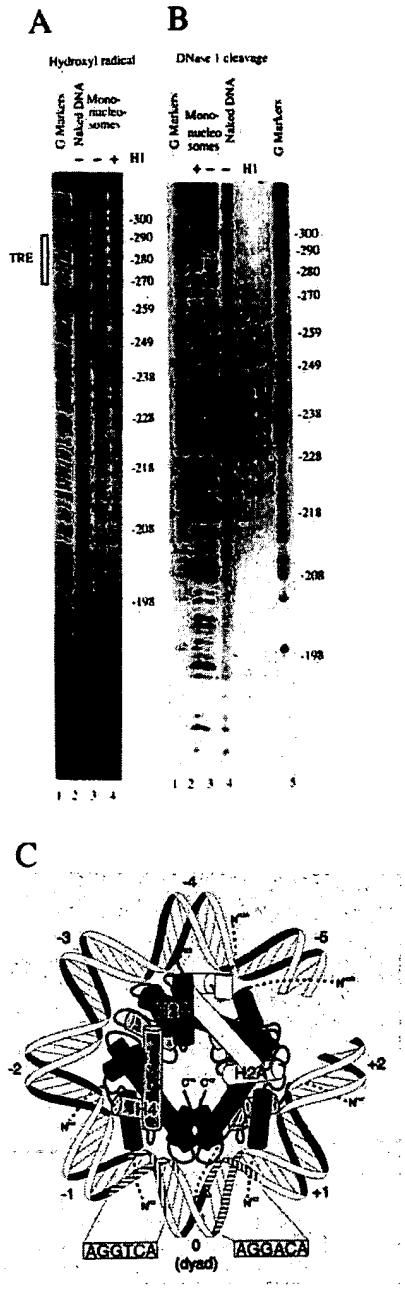

Fig. 3. Hydroxyl radical and DNase I footprinting of mononucleosome in the presence or absence of histone H1. Individual complexes were isolated by non-denaturing agarose (0.7%) gel electrophoresis after hydroxyl radical or DNase I treatment. DNA from these complexes was isolated and analyzed by denaturing polyacrylamide (6%) gel electrophoresis. (**A**) Hydroxyl radical cleavage of mononucleosomes. Lane 1, G sequencing markers; lane 2, cleavage of naked DNA; lanes 3 and 4, cleavage of nucleosomal DNA in the absence or presence of histone H1 respectively. The approximate position of the TRE is indicated. The markers indicate base pair positions relative to the start site of transcription (+1). (**B**) DNase I cleavage of mononucleosomes. Lanes 1 and 5, G sequencing markers; lanes 2 and 3, cleavage of nucleosomal DNA in the presence or absence of histone H1 respectively. Lane 4 shows the cleavage of naked DNA. (**C**) A model for the nucleosome assembled on the TRβA gene that contains the TRE. The TRE is indicated by the hatched DNA and the boxed sequences. The dyad position in the nucleosome is indicated as are integral turns of DNA away from the dyad axis (negative to 5′, positive to 3′). The histones are shown in the cartoon (see Arents *et al.*, 1991; Arents and Moudrianakis, 1993; Pruss *et al.*, 1995 for details of histone positions).

TR/RXR on naked DNA (Glass, 1994). The arrangement of the histones is based on the octamer structure proposed by Moudrianakis and colleagues (Arents *et al.*, 1991; Arents and Moudrianakis, 1993), protein–DNA crosslinking (Pruss and Wolffe, 1993; Pruss *et al.*, 1996) and nuclease mapping studies of histone–DNA interactions (this work; Hayes and Wolffe, 1993; reviewed by Pruss *et al.*, 1995).

The incorporation of histone H1 into the nucleosome does not lead to any additional protection of DNA from hydroxyl radical cleavage. This result is in agreement with earlier work on mononucleosome templates (Hayes and Wolffe, 1993; Nightingale *et al.*, 1996a). Moreover, inclusion of histone H1 into the nucleosome does not influence DNase I cleavage (Figure 3B, compare lanes 2 and 3). This result is also in agreement with earlier work (Hayes and Wolffe, 1993; Nightingale *et al.*, 1996a). We conclude that the nucleosome including the TRE of the TRβA gene is highly positioned both with respect to the translational positioning of histone–DNA contacts and with respect to the rotational positioning of DNA on the core histone surface.

The influence of histone H1 on TR/RXR interaction with the TRE in vitro and on transcriptional control of the TRβA promoter following microinjection into Xenopus oocyte nuclei

Our structural studies indicate that the inclusion of histone H1 into the TRβA nucleosome has no influence on hydroxyl radical or DNase I cleavage of DNA. This suggests that even in the presence of histone H1, the TRE at the nucleosomal dyad should remain accessible to TR/RXR. A model for the nucleosome in which there is an asymmetric binding of the winged-helix domain of histone H1 inside the gyres of DNA in the nucleosome (Pruss *et al.*, 1996) could account for the lack of protection by H1 to DNase I cleavage (Figure 3) and the asymmetric protection by H1 from micrococcal nuclease (Figure 2). We next examined whether inclusion of histone H1 into the nucleosome would influence the specific interaction of TR/RXR with the TRE in the nucleosome. Consistent with earlier work (Wong *et al.*, 1995), the TR/RXR binds to the TRE associated with a histone octamer (Figure 4A, compare lanes 1 and 2). This binding is specific, because it is competed by a wild-type TRE (lane 3, wt) but not by a mutant TRE (lane 4, mt). The addition of histone H1 retards the nucleosome complex in the non-denaturing gel (Figure 4A, compare lanes 1 and 5), yet does not prevent the specific binding of the TR/RXR to the nucleosome (Figure 4A, lanes 6–8). Comparison of lanes 5 and 6 in Figure 4A suggests that histone H1 might be displaced on addition of TR/RXR. In order to better examine this possibility we first increased the amount of histone H1 added to the binding reaction, and to avoid non-specific aggregation of nucleoprotein complexes under these conditions we increased the amount of non-specific competitor (Figure 4B). Comparison of lanes 1 and 5 in Figure 4B shows a clear association of histone H1 with the nucleosomal template. This association is substantially lost on addition of TR/RXR (Figure 4B, compare lane 5 with lanes 6–8). Addition of TR/RXR and/or the addition of the competitor TRE oligonucleotide, but not oocyte extract alone (lane 5) appear to displace histone H1 from

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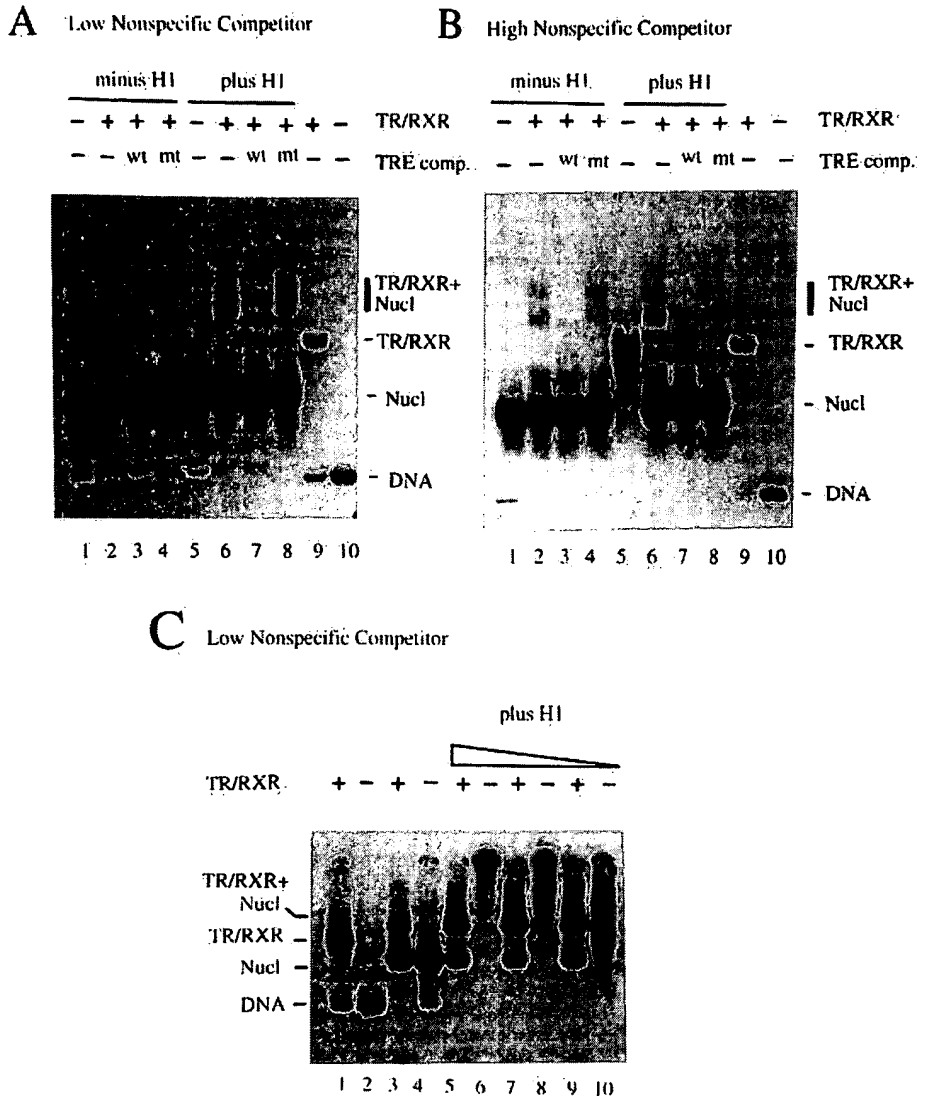

Fig. 4. TR/RXR binding to the TRE in the nucleosome in the absence or presence of histone H1. (**A** and **B**) Two sets of identical gel retardation experiments are shown with the major differences being the mass of histone H1 added, equimolar amounts in (A) and a 2-fold molar excess relative to nucleosome cores of H1 in (B) and the excess of non-specific DNA per binding reaction being 50 ng of dI.dC in (A) and 500 ng in (B). In (A), the nucleosomes were assembled as described in Figure 1B; lane 1, minus H1; lane 5, plus H1. In (B), the nucleosomes were reconstituted as described in Figure 1B; lane 1, minus H1; lane 6, plus H1. TR/RXR was added in the oocyte extract (2 μl) as indicated (+) or oocyte extract alone was added (–) in the absence or presence of 10 ng wild-type or mutant TRE oligonucleotide competitor as indicated. Lane 9 contains naked TRE containing TRβA gene fragment and 2 μl of oocyte extract containing TR/RXR. Lane 10 contains the DNA probe alone (see Materials and methods). The position of free DNA (DNA), nucleosomal DNA (Nucl), TR/RXR-bound DNA (TR/RXR) and TR/RXR-bound nucleosome (TR/RXR + Nucl) are indicated. The reason for a doublet in the TR/RXR + Nucl complex is potentially due to the binding of a second TR/RXR to a cryptic TRE as the higher concentration of poly dI.dC [shown in (B)] reduces the upper band. (**C**) In this experiment higher concentrations of TR/RXR and histone H1 are used as indicated below. An excess of 50 ng of dI.dC is used as non-specific competitor. Reconstitutions of nucleosome cores was as in Figure 1A: all lanes contain 100 mg of cores except lanes 1 and 2 which contain 100 ng of naked DNA (= 1.2 pmol). Lanes 9 and 10 contain 1.8 pmol of H1, lanes 7 and 8 contain 2.4 pmol of H1, and lanes 5 and 6 contain 3.0 pmol of H1. TR/RXR was added in the oocyte extract (5 μl) as indicated (+) or oocyte extract alone was added (–). The position of free DNA (DNA), nucleosomal DNA (Nucl), TR/RXR-bound DNA (TR/RXR) and TR/RXR-bound nucleosome (TR/RXR + Nucl) are indicated.

the nucleosome in the presence of high concentrations of non-specific competitor DNA. Our second approach was to maintain the low non-specific competitor concentrations, and to increase the concentration of both TR/RXR and histone H1 in the binding reaction. These latter conditions have the advantage that a more substantial association of the receptor with nucleosomal DNA is achieved (Figure 4C, lanes 3, 5, 7 and 9). Thus, the competition between the receptor and histone H1 is more pronounced (Figure 4C, compare lane 5 with 6, lane 7 with 8, and lane 9 with 10). In every case, even under conditions where histone H1 would direct substantial aggregation of nucleosomes (lanes 6 and 8), the addition of TR/RXR appears to displace histone H1 from the nucleosome (lanes 5 and 7).

The fact that the prior inclusion of histone H1 into the nucleosome does not prevent the association of the TR/RXR with nucleosomal DNA (Figure 4A, lanes 5 and 6; Figure 4B, lanes 5 and 6; Figure 4C, lanes 5–10), led us to investigate the consequences of histone H1 expression on the transcription of the TRβA promoter following

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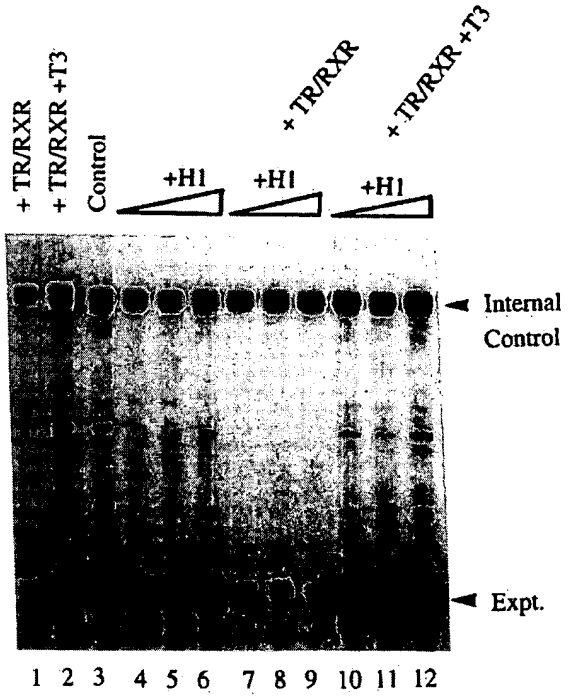

Fig. 5. The influence of histone H1 expression on the regulation of TRβA gene expression in oocytes. Groups of 20 oocytes were injected without or with histone H1C mRNA (0.9 ng/oocyte) as indicated. After 6 h the oocytes were injected with double-stranded pTRβA either with or without TRβ/RXRα mRNAs (0.9 ng/oocyte). The oocytes were then treated for 16 h with or without T_3 as indicated. The RNA transcribed by TRβA was analyzed. The mRNA initiated at the TRβA promoter is shown (Expt.) together with an internal control (Internal Control).

microinjection into *Xenopus* oocytes. It should be noted that oocytes do not contain histone H1; however, if H1 mRNA is injected into the oocyte cytoplasm, the protein is synthesized, taken up into the nucleus and incorporated into chromatin (Shimamura *et al.*, 1989; Bouvet and Wolffe, 1994; Bouvet *et al.*, 1994; P.Bouvet, S.Dimitrov and A.P.Wolffe, unpublished observations). In earlier work we demonstrated that following microinjection of the TRβA promoter as double-stranded DNA, high levels of basal transcription activity were obtained (Wong *et al.*, 1995, 1997). The injection of mRNA encoding both components of the heterodimer TR/RXR led to the synthesis of the receptor, which in the absence of thyroid hormone associates with chromatin and silences transcription (Figure 5, compare lanes 1 and 3; Sap *et al.*, 1989; Wong *et al.*, 1995). Addition of thyroid hormone relieves transcriptional silencing, returning transcription activity back to basal levels (Figure 5, compare lanes 1–3; Barettino *et al.*, 1994; Wong *et al.*, 1995). The expression of histone H1 following the injection of histone H1 mRNA into the oocyte cytoplasm leads to a weak (~2-fold) repression of basal transcription (Figure 5, compare lane 3 with lanes 4–6). Interestingly, increasing amounts of histone H1 are less repressive; this may be related to an increase in nucleosome spacing concomitant with histone H1 incorporation into chromatin (Shimamura *et al.*, 1989; Sandaltzopoulos *et al.*, 1994) and the observation that inclusion of more than a single molecule of histone H1 per nucleosomal repeat results in a lower binding affinity of H1 for chromatin (Nightingale *et al.*, 1996a). Histone H1 does not prevent TR/RXR from silencing transcription in the absence of thyroid hormone (Figure 5, compare lane 3 with lanes 7–9). Moreover, the subsequent addition of thyroid hormone relieves the repressive and silencing effects attributed to histone H1 and the unliganded thyroid hormone receptor respectively (Figure 5, lanes 10–12). Thus, consistent with the lack of effect of histone H1 on the association of TR/RXR with the TRE in a nucleosome (Figure 4), histone H1 has no major influence on transcription of the TRβA promoter following microinjection into *Xenopus* oocyte nuclei (Figure 5).

A role for the rotational positioning of the TRE in both binding of TR/RXR to nucleosomal DNA and the transcriptional response to thyroid hormone

The precise rotational positioning of the TRE within a translationally positioned nucleosome (Figures 2 and 3) led us to consider whether this level of nucleosome organization was important for the binding of TR/RXR and the transcriptional regulation of the TRβA gene. Our first experiments attempted to alter the rotational positioning of the TRE by the deletion of 3, 6 and 10 base pairs within the sequence +251 to +261 relative to start site of transcription (+1) in the sequence immediately adjacent to the TRE (Figure 6). This approach failed to alter the major rotational position of the TRE on the surface of the nucleosome (Figure 6, lanes 10–13, dotted region indicates the TRE). However, for one of the mutant constructs, the quality of modulation in hydroxyl radical cleavage of the reconstituted nucleosome was severely reduced (Figure 6, lane 12, compare with lanes 10, 11 and 13). This might be explained by the presence of several possible rotational positions for DNA on the surface of the histone octamer (Roberts *et al.*, 1995). The maintenance of a predominant rotational positioning of DNA in the nucleosome indicates that the features of intrinsic DNA sequence and structure that determine rotational position lie 3′ of +261 in the TRβA gene. Deletion of sequences 5′ of +261 will therefore alter the translational position of the TRE relative to the start site of transcription (+1) within chromatin. The region 3′ of +261 shows a strong modulation of hydroxyl radical cleavage even as naked DNA (Figure 6, lanes 6–9, see upper portion of gel lanes). This modulation indicates a variation in minor groove width in the double helix and the potential for DNA structures that could influence nucleosome positioning (Hayes *et al.*, 1990, 1991). The variation in quality of rotational positioning of DNA on the surface of the histone octamer (Figure 6, compare lanes 10, 11 and 13 with lane 12), led us to examine the consequence for binding of the TR/RXR to nucleosomal DNA, the consequences for transcriptional activation in response to thyroid hormone and the disruption of chromatin structure (Wong *et al.*, 1995).

Comparison of the association of TR/RXR with the TRE in nucleosomes containing either the wild-type [pTRβA(p)] gene or the mutants in which rotational positioning is maintained [pTRβA(p)-3] or perturbed [pTRβA(p)-6] demonstrates that the loss of precise rotational phasing is without significant consequence (Figure 7A, compare lanes 9–12 with lanes 1–8). A clear tertiary complex of TR/RXR, histones and DNA is obtained (R·N

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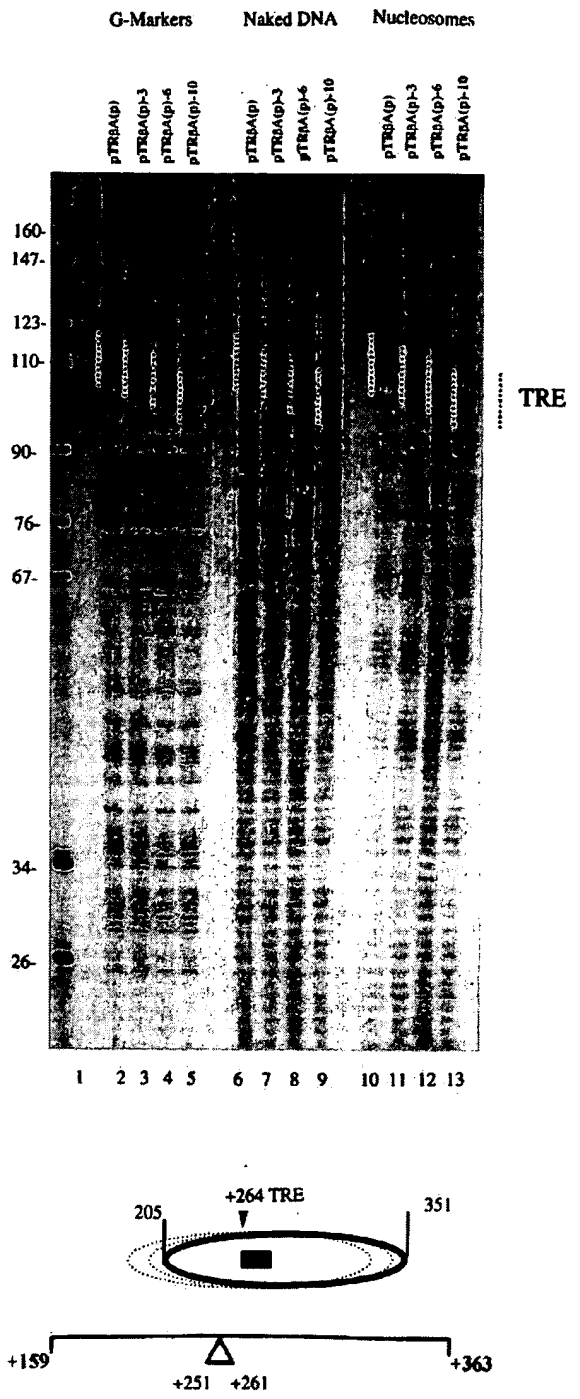

Fig. 6. The effect of deletions of sequences flanking the TRE on rotational positioning relative to the histone surface. Either wild-type TRβA gene sequences [pTRβA(p)] or mutations of the gene where three [pTRβA(p)-3], six [pTRβA(p)-6] or ten [pTRβA(p)-10] base pairs were deleted to the 3′ of +251 (relative to the start site of transcription), were cleaved either with Maxam and Gilbert chemistry (G-Markers, lanes 2–5), with the hydroxyl radical as naked DNA (Naked DNA, lanes 6–9) or with the hydroxyl radical as nucleosomal DNA (Nucleosomes, lanes 10–13) before resolution on an 8% denaturing polyacrylamide gel. Markers are from pBR322 digested with *Msp*I. The exact position of the TRE is indicated in each lane by the left-hand dotted line. The diagram in the lower panel shows the approximate position of the TRE and the mutations together with major and minor translational positions of the histone octamer (see Figure 2).

in lanes 4, 8 and 12). We further examined the association of TR/RXR with these nucleosomes under conditions such that a more substantial fraction of the nucleosomal reconstitutes were bound by TR/RXR (Figure 7B). We again find that the binding of TR/RXR to nucleosomal DNA is not influenced by the loss of precise rotational position or by the concomitant changes in translational position (Figure 7B, compare lanes 7–9 with lanes 1–6). We next examined the transcriptional response of the mutant promoters. Wild-type and mutant TRβA promoters are assembled into a repressive chromatin structure following replicative chromatin assembly during second strand DNA synthesis on single-stranded templates microinjected into oocyte nuclei (Figure 7C, lanes 1, 7 and 13; Wong *et al.*, 1995). Addition of TR/RXR and thyroid hormone relieves this transcriptional repression to an equivalent extent independent of the mutations flanking the TRE (Figure 7B). Consistent with the activation of transcription being a property of the vast majority of minichromosome templates in the nucleus, all of the templates show an alteration of DNA topology consistent with nucleosome disruption following the addition of thyroid hormone in the presence of TR/RXR (Figure 7D, compare lanes 3, 6 and 9 with the other lanes; see Wong *et al.*, 1997). This assay relies upon each nucleosome introducing a single negative superhelical turn into a closed circular plasmid molecule in the presence of topoisomerase I (Germond *et al.*, 1975; Simpson *et al.*, 1985). Disruption of nucleosomes associated with transcriptional activation leads to the observed changes in DNA topology on the TRβA constructs (Wong *et al.*, 1997). Consistent with the equivalent transcriptional regulatory properties, mapping of DNase I hypersensitivity in the assembled minichromosome by indirect end-labeling (Figure 7C) reveals that TR/RXR binds to the TRE within chromatin with very similar preferences to each of the three templates. Thus, the association of TR/RXR with nucleosomal DNA is independent of the loss of precise rotational positioning or small alterations in translational position (Figure 7C, compare lanes 12–15 with lanes 4–11). Comparison of Figure 7C, lanes 1–3 which show the DNase I cleavage of minichromosomes assembled in the oocyte in the absence of TR/RXR, with Figure 7C, lanes 4–15 in which TR/RXR has been expressed, shows that the formation of the DNase I-hypersensitive site is dependent on the receptor. We conclude that neither the loss of precise rotational positioning of the TRE nor small alterations in the translational position of the TRE by 3 and 6 bp interfere with the capacity of TR/RXR to regulate transcription in a chromatin environment. We next attempted to fix the rotational positioning of the TRE such that it would be in a deliberately unfavorable orientation relative to the histone surface for efficient association of TR/RXR.

Our strategy to fix the rotational positioning of the TRE in an unfavorable orientation was to fuse it to a DNA sequence that is known independently to direct strong rotational positioning of the double helix on the surface of the histone octamer. We made use of the *X.borealis* somatic 5S rRNA gene which interacts with core histones to direct the assembly of a strongly positioned nucleosome (Hayes *et al.*, 1990; Hayes and Wolffe, 1993). We have used this sequence in earlier experiments to alter the rotational positioning of the adenovirus major late pro-

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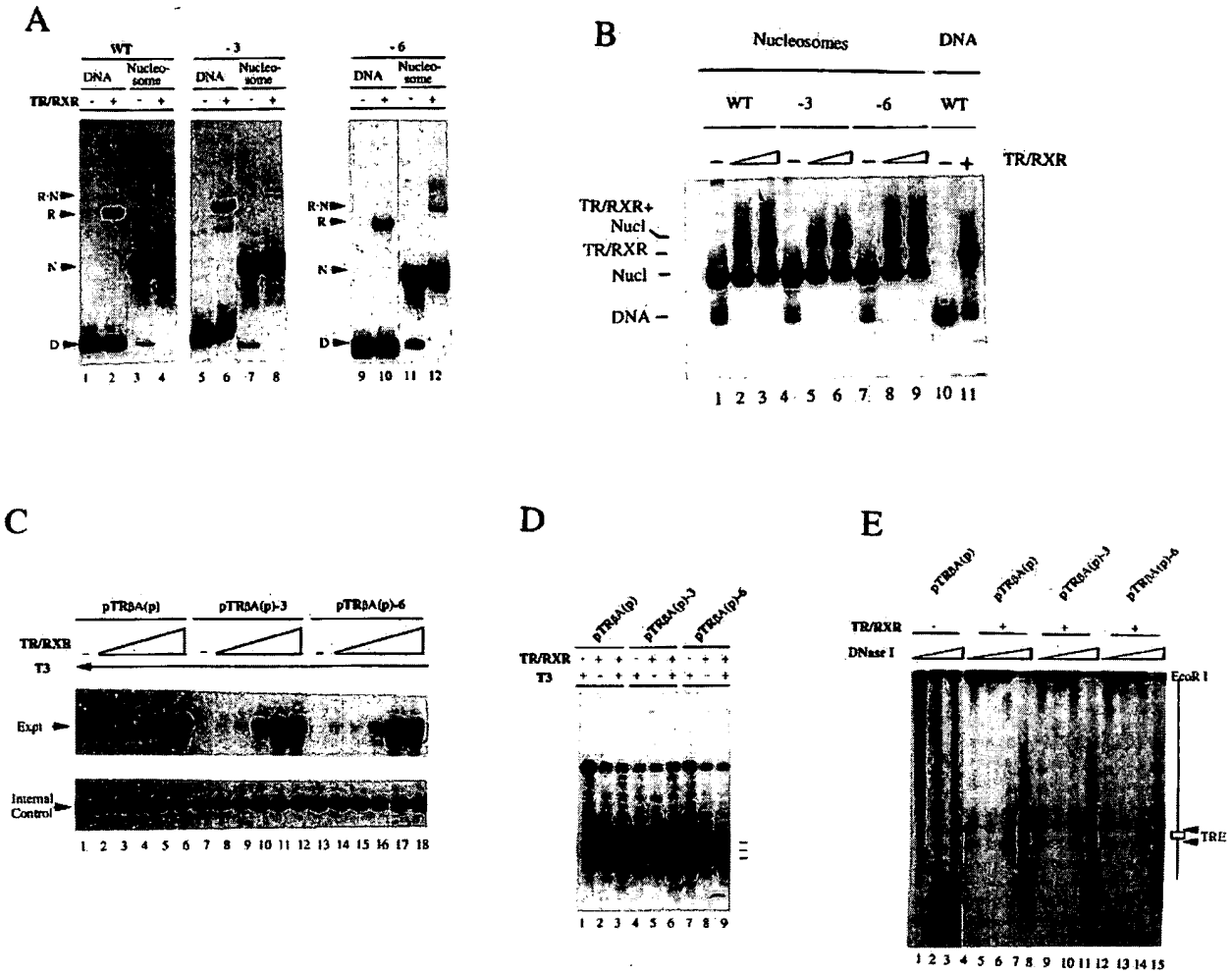

Fig. 7. Variation in the quality of rotational positioning is without significant effects on the binding of TR/RXR to nucleosomal DNA, transcriptional activation and chromatin disruption. (**A**) DNA fragments derived from the wild-type TRβA gene (WT) and the mutants in which 3 bp (–3) and 6 bp (–6) were deleted were reconstituted into nucleosome cores and the binding of TR/RXR assayed as described for Figure 4A. Lanes 1, 5 and 9 show naked DNA; lanes 2, 6 and 10 show naked DNA bound by TR/RXR; lanes 3, 7 and 11 show nucleosomal DNA; and lanes 4, 8 and 12 TR/RXR bound to nucleosomal DNA. The positions of naked DNA (D), nucleosome (N), TR/RXR–DNA complex (R) and TR/RXR–nucleosome complex (R·N) are indicated. (**B**) Binding of TR/RXR to WT, –3 and –6 nucleosomes under the conditions described in Figure 4C. Lanes 1–9 contain nucleosome cores, lane 10 and 11 contain naked DNA. In lanes 1, 4, 7 and 10, 5 μl of oocyte extract in which TR/RXR had not been expressed was added to the binding reaction. In lanes 2, 5, 8 and 11, 5 μl of oocyte extract in which TR/RXR had been expressed were added; in lanes 3, 6 and 9, 8 μl of oocyte extract containing TR/RXR have been added. The positions of free DNA (DNA), nucleosomal DNA (Nucl), TR/RXR-bound DNA (TR/RXR) and TR/RXR-bound nucleosome (TR/RXR + Nucl) are indicated. (**C**) *Xenopus* oocytes were microinjected without (–) or with increasing masses of TR/RXR mRNAs (0.1, 0.3, 0.5, 0.7 and 0.9 ng) as indicated by the triangles. After 6 h, single-stranded TRβA wild-type or mutant templates were microinjected. The oocytes were then treated for 16 h with T_3 as indicated. Transcripts from the TRβA (Expt) and an internal control (Internal Control) are shown. (**D**) In parallel with the experiment shown in (C) the topology of the template DNA was examined at the end of the incubation period. In this experiment 0.9 ng of TR/RXR mRNAs were injected or not as indicated. T_3 was present or absent from the incubation medium as indicated. The lines at the right-hand side of the figure indicate the average change of three in the linking number of DNA observed for minichromosomes in the presence of TR/RXR dependent on the absence or presence of T_3, following resolution on a 1% agarose gel containing chloroquine (see Materials and methods). (**E**) In parallel with the experiments shown in (C) and (D), the binding of TR/RXR to the TRE in the context of a polynucleosomal array was assayed by DNase I-hypersensitive site analysis. In this experiment, 0.9 ng of TR/RXR mRNAs were injected (+) or not (–) as indicated. T_3 was absent from the incubation medium in this experiment. The DNase I concentrations were 10 U in lanes 4, 8 and 12; 20 U in lanes 1, 5, 9 and 13; 40 U in lanes 2, 6, 10 and 14; and 80 U in lanes 3, 7, 11 and 15. DNA was cleaved with *Eco*RI and probed with a radiolabeled CAT DNA fragment abutting the *Eco*RI site (see Materials and methods). The positions of *Eco*RI sites and the TRE are indicated.

moter TATA box with respect to the histone surface (Godde *et al.*, 1995). Sequences from +154 to +257 (103 bp) within the TRβA gene were replaced with either a 94 bp or 97 bp DNA fragment including the 5S rRNA gene (Figure 8A). This has the consequence of moving the TRE from +264 in the wild-type TRβA promoter to either +255 (5S-A) or +258 (5S-B) relative to the start site of transcription. A second consequence is that the 5S rRNA nucleosome positioning predominates over that of the TRE, with the TRE altering rotational position by 3 bp with respect to the histone surface (Figure 8B, compare lanes 1–4). Comparison with the wild-type orientation leads to the conclusion that the TRE in the 5S-B construct has a rotational position more similar to wild-type than the 5S-A construct (Figure 8B, compare lanes 5–8; note that lanes 5 and 6 are from a different portion

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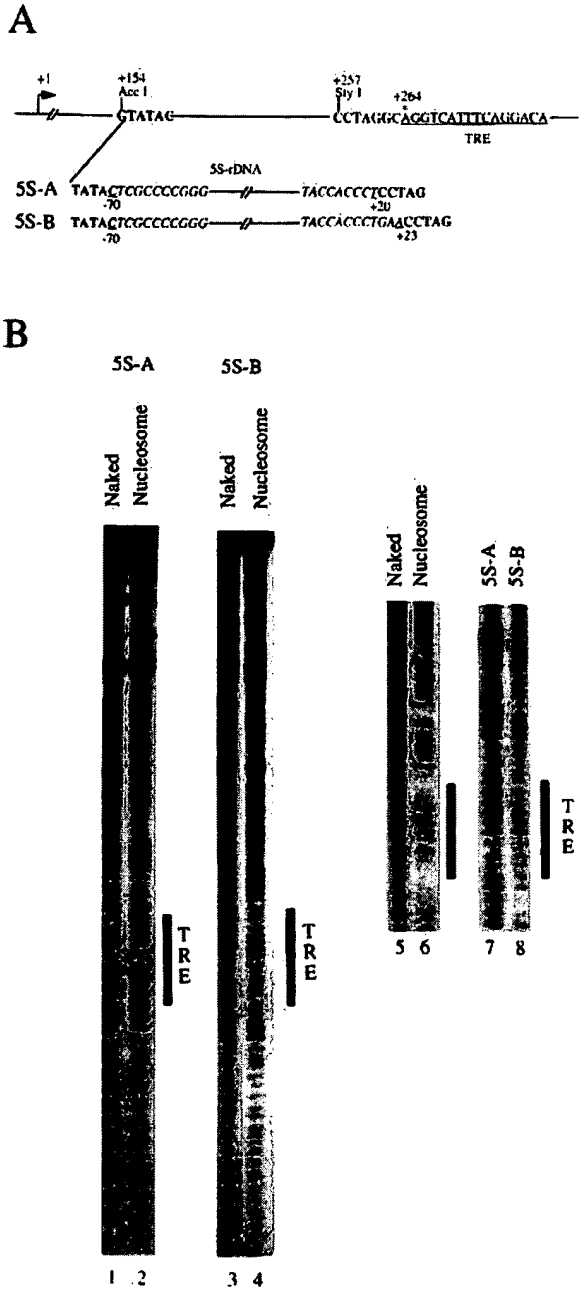

Fig. 8. Altering rotational position of the TRE using *X.borealis* 5S rRNA gene sequences. (**A**) In order to alter the rotational positioning of the TRE relative to the histone surface we replaced TRβA gene sequences with those of the *X.borealis* somatic 5S rRNA gene as indicated (see Materials and methods). These DNA fragments were then reconstituted into nucleosomes and the rotational positions of the TRE determined. The asterisk indicates the start of the TRE. (**B**) Hydroxyl radical cleavage of naked DNA for 5S-A (lane 1), for 5S-B (lane 3) and for wild-type (lane 5), and hydroxyl radical cleavage of nucleosomal DNA for 5S-A (lanes 2 and 7), for 5S-B (lanes 4 and 8) and for wild-type (lane 6) is shown. The position of the TRE is indicated for each construct. Note that the images in lanes 5 and 6 and 7 and 8 come from different portions of the gel.

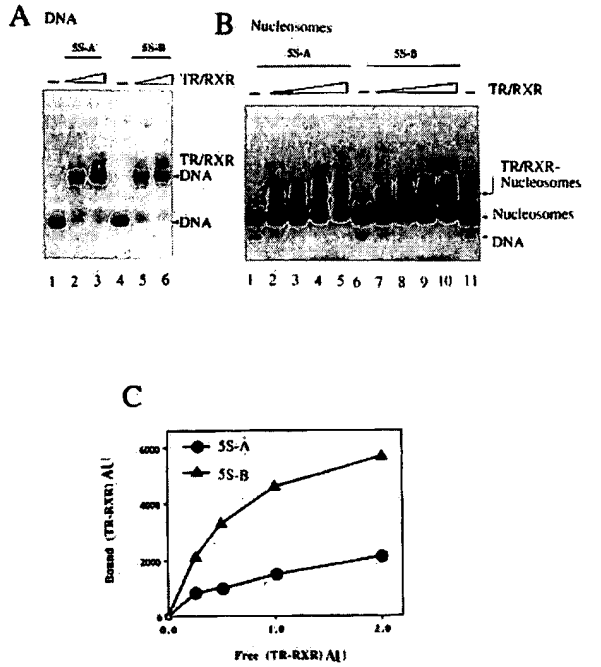

Fig. 9. The influence of altering rotational positioning of the TRE on the binding of TR/RXR to nucleosome DNA *in vitro*. (**A**) DNA fragments of the 5S-A and 5S-B constructs were bound by 5 (lanes 2 and 5) and 10 μl (lanes 3 and 6) of oocyte extract containing TR/RXR. Lanes 1 and 4 contain 5 μl of oocyte extract lacking TR/RXR. (**B**) DNA fragments from the 5S-A and 5S-B constructs were reconstituted with histone octamers and the binding of TR/RXR assayed as described for Figure 4C. Lanes 1 and 6 contain no extract; lanes 2 and 7, 0. 5 μl; lanes 3 and 8, 1 μl, lanes 4 and 9, 2 μl; and lanes 5 and 10, 10 μl of oocyte extract containing TR/RXR. The positions of DNA (DNA) the nucleosome (Nucleosomes) and the TR/RXR–nucleosome complex (T/RXR-Nucleosomes) are indicated. (**C**) Quantitation of the data shown in (B).

of the gel from lanes 7 and 8). Our next experiments examined the functional consequences of moving the rotational position of the TRE by 3 bp. We find that the TR-RXR binds equivalently to the two naked DNA sequences (Figure 9A, lanes 1–6); however, TR-RXR binds significantly better to the 5S-B sequence than to the 5S-A sequence in a nucleosomal context (Figure 9B, lanes 1–11). Quantitation of bound TR/RXR using the phosphorimager indicates that the TR/RXR prefers to associate with the 5S-B nucleosome by 2- to 3-fold relative to the 5S-A nucleosome (Figure 9C). We find hormone-bound receptor activates transcription at least 2-fold more effectively from the 5S-B construct in chromatin than from the 5S-A construct (Figure 10A, compare lanes 1–6 with lanes 7–12), dependent on an increase in expression of the receptor (Figure 10B). In earlier work we have documented that hormone-bound TR/RXR will disrupt nucleosomes as assayed by topological changes in minichromosomes dependent on the association of the receptor with the TRE in chromatin (Wong *et al.*, 1995, 1997). We find that the capacity of TR/RXR to disrupt nucleosomes in minichromosomes is significantly reduced for the 5S-A minichromosome relative to the 5S-B minichromosome (Figure 10C, lanes 1–4). This result is consistent with the relative transcriptional activities of the 5S-A and 5S-B minichromosomes (Figure 10A, lanes 1–12). Mapping of DNase I hypersensitivity in the assembled minichromosome by indirect end-labeling (Figure 10D) reveals that TR/RXR binds to the TRE within chromatin more effectively to the 5S-B construct than to the 5S-A construct (Figure 10D, compare lanes 4–6 with lanes 7–9). Finally,

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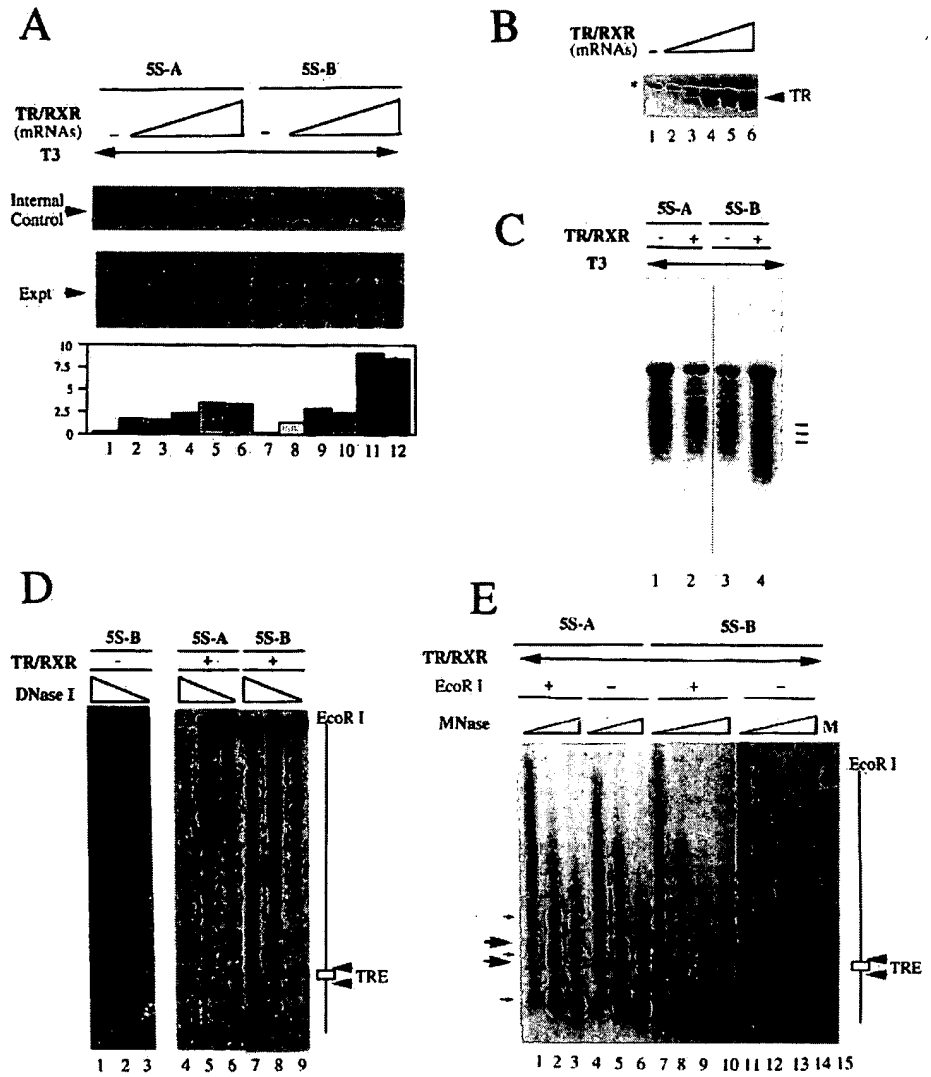

Fig. 10. (**A**) *Xenopus* oocytes were microinjected without (–) or with masses of TR/RXR mRNAs (0.1, 0.3, 0.5, 0.7 and 0.9 ng) as indicated by the triangles. After 6 h, single-stranded 5S-A or 5S-B templates were microinjected. The oocytes were then incubated for 16 h with T_3 as indicated. Transcripts from the 5S-A and 5S-B (Expt) and an internal control (Internal Control) are shown. The lower panel shows a quantitation of the Expt transcript. (**B**) An immunoblot showing the abundance of thyroid hormone receptor (TR, indicated by the arrowhead) synthesized under the injection conditions used in (A). The asterisk indicates an endogenous non-specific cross-reactive protein that is not TR. (**C**) In parallel with the experiment shown in (A), the topology of the template DNA was examined at the end of the incubation period. In this experiment, 0.9 ng of TR/RXR mRNAs were injected into all the oocytes. T_3 was present (+) or not (–) as indicated. The lines at the right-hand side of the figure indicate the average change of three in the linking number of DNA observed for minichromosomes in the presence of TR/RXR and a TRE dependent on the absence or presence of T_3, following resolution on a 1% agarose gel containing chloroquine (see Materials and methods). (**D**) Groups of 20 oocytes were injected with or without TR/RXR mRNAs (0.9 ng/oocyte) and ssDNA of 5S-A or 5S-B, and treated with or without T_3 as indicated. After an overnight incubation the oocytes were collected for the DNase I sensitivity assay. The DNase I concentrations were 80 (lanes 4 and 7), 40 (lanes 5 and 8) and 20 U (lanes 6 and 9). DNA was cleaved with *Eco*RI and probed with a radiolabeled CAT DNA fragment abutting the *Eco*RI site (see Materials and methods). The positions of *Eco*RI sites and the TRE are indicated. (**E**) Groups of 20 oocytes were injected with TR/RXR (0.9 ng/oocyte) and ssDNA of 5S-A or 5S-B in the absence of T_3. After an overnight incubation the oocytes were collected for the micrococcal nuclease mapping of nucleosome positions. The micrococcal nuclease concentrations were 1.25 U (lanes 7 and 11), 2.5 U (lanes 1, 4, 8 and 12), 5U (lanes 2, 5, 9 and 13) and 10 U (lanes 3, 6, 10 and 14). Lane 15 shows markers of 344, 396, 506 bp and 1.9 kb. The DNA was either digested (+) or not (–) with *Eco*RI as indicated. The small arrow heads on the left of the panel indicate the position of mono-, di- and trinucleosomes. The large arrow heads indicate the region of perturbation of MNase cleavage in lanes 7–10 relative to lanes 1–3. The positions of the *Eco*RI site and the TRE are indicated.

we mapped micronuclease cleavage sites in the 5S-A and 5S-B minichromosomes. We find that both templates are assembled into nucleosomal arrays (Figure 10E, lanes 4–6 and 11–14). Indirect end-labeling following restriction endonuclease cleavage reveals considerable nucleosome positioning on these constructs, as might be anticipated from earlier observations (Wolffe and Drew, 1989; Hayes *et al.*, 1990). The micrococcal nuclease cleavage sites show alterations at the TRE in the 5S-B nucleosome (Figure 10E, lanes 7–10, large arrow heads) that are much less pronounced in the 5S-A nucleosome (Figure 10E, lanes 1–3). Our results (Figure 10D and E) are consistent with a greater occupancy of the TRE with the TR/RXR in a polynucleosomal array in the 5S-B minichromosome

relative to the 5S-A minichromosome. We conclude that small changes in the rotational position of the TRE can influence binding of the TR/RXR to chromatin *in vivo* and *in vitro* with related changes in the efficiency of transcriptional regulation.

Discussion

The major conclusions from these experiments are: (i) that the TRβA gene sequences downstream from the start site of transcription have the capacity to direct nucleosome positioning (Figures 1–3); (ii) that incorporation of histone H1 into the nucleosome including the TRE leads to an asymmetric extension of histone–DNA contacts relative to the nucleosome core, but is without influence on the binding of TR/RXR (Figures 1–5); and (iii) that the rotational positioning of the TRE on the surface of the nucleosome can influence TR/RXR binding *in vitro* and transcription *in vivo* (Figures 6–10).

Nucleosome positioning and transcriptional control

Several acutely inducible promoters are assembled into highly organized chromatin structures including positioned nucleosomes. These include the *Saccharomyces cerevisiae PHO5* promoter (Almer and Horz, 1986; Almer *et al.*, 1986), the mouse mammary tumor virus long terminal repeat (MMTV LTR; Richard-Foy and Hager, 1987) and the human immunodeficiency virus long terminal repeat (HIV-1 LTR; Verdin *et al.*, 1993). The determinants of nucleosome positioning have not been completely described; however, it is known that the exact sequences assembled into nucleosomes can be important for the transcriptional response (Straka and Horz, 1991). The translational positioning of nucleosomes (Truss *et al.*, 1995; Chavez and Beato, 1997) and the rotational positioning of DNA on the surface of the histones (Perlmann and Wrange, 1988; Pina *et al.*, 1990a,b; Archer *et al.*, 1991) have been suggested as important contributory factors in the regulation of MMTV LTR transcription by the glucocorticoid receptor. Nevertheless, the exact nature and stability of histone–DNA contacts on the MMTV LTR *in vivo* are yet to be completely resolved (Fragoso *et al.*, 1995; Roberts *et al.*, 1995). *In vitro* experiments have shown that both the rotational positioning and translational positioning of the glucocorticoid receptor recognition element within a nucleosome can influence the binding of the receptor (Li and Wrange, 1993, 1995). The structure of the nucleosome containing the TRE within the TRβA gene sequence could therefore be important for transcriptional control.

The DNA sequences within the first 500 bp of the TRβA gene have the capacity to direct the translational positioning of histone octamers (Figure 2). Although we have not systematically manipulated the translational position of the histone octamers with respect to the TRE and the transcription start site, moving the translational position of the TRE within chromatin by 3 and 6 bp (Figures 6 and 7) with relatively minor alterations in rotational positioning of the TRE is without major effects on the transcriptional response to TR/RXR in the presence of thyroid hormone (Figure 7).

The rotational position adopted by the TRE on the surface of the nucleosome assembled on the wild-type TRβA gene appears optimal for continued access of the TR/RXR within chromatin. The key contacts made by the receptor with the TRE through the major groove are predicted to remain accessible from solution (Figure 3; Glass, 1994). This accounts for the continued capacity of TR/RXR to bind the TRE in the nucleosome (Figure 4). A small 3 bp alteration in the rotational positioning of the TRE with respect to the histone surface leads to a reduction in the capacity of TR/RXR to bind to nucleosomal DNA *in vitro* (Figures 9), to a reduction in the capacity of TR/RXR to bind to polynucleosomal arrays *in vivo* (Figure 10C, D and E), and to a reduction in the capacity of TR/RXR to activate transcription (Figure 10A and B). These results, especially when coupled to other manipulations that alter translational position but lead to no significant alterations either in TR/RXR binding to nucleosomal DNA or in transcriptional responsiveness, indicate that the rotational positioning of DNA within the nucleosome can contribute to the transcriptional response.

Histone H1, the nucleosome and transcriptional control

In earlier work we have made use of the *Xenopus* 5S rRNA genes to examine the structural and functional consequences of incorporating linker histones such as histone H1 into chromatin. Our conclusions were that the inclusion of histone H1 directed an asymmetric protection of DNA flanking the nucleosome core from cleavage with micrococcal nuclease (Hayes and Wolffe, 1993; Ura *et al.*, 1995), that the winged-helix domain of histone H1 occupied an asymmetric position within the nucleosome (Hayes *et al.*, 1994; Pruss *et al.*, 1996; see also Hayes, 1996), and that incorporation of histone H1 could direct the transcriptional repression of 5S rRNA genes (Ura *et al.*, 1995, 1997). There are several similarities, but also differences, concerning the consequences of incorporating histone H1 into nucleosomes using the TRβA gene.

Incorporation of histone H1 into the TRβA gene nucleosome that contains the TRE leads to an asymmetric protection of DNA sequences flanking the nucleosome core from micrococcal nuclease cleavage (Figure 2A, lanes 1–10). A comparable asymmetric protection of DNA sequence occurs when histone H1 is incorporated into the adjacent nucleosome (Figure 2B, lanes 1–8). These results are similar to those obtained with the *X.borealis* 5S rRNA gene (Hayes and Wolffe, 1993; Ura *et al.*, 1995). They lead to the suggestion that an asymmetric association of linker histones within the nucleosome (Pruss *et al.*, 1996) may be a relatively common feature of nucleosomal architecture (see Crane-Robinson, 1997). Consistent with the model for the 5S rRNA gene nucleosome (Pruss *et al.*, 1996), incorporation of histone H1 leads to no significant changes in hydroxyl radical or DNase I cleavage of nucleosomal DNA (Figure 3). This would be anticipated if the winged-helix domain of histone H1 were positioned inside the gyres of DNA in the nucleosome and not at the dyad axis (Crane-Robinson, 1997). If the winged-helix domain of histone H1 occupies a position away from the dyad axis of the nucleosome then it would not be expected to impede access of TR/RXR to the TRE which itself occupies the dyad axis of the nucleosome (Figure 3). However, although TR/RXR can bind effectively to a

nucleosome containing histone H1 (Figure 4), it displaces histone H1 as a consequence of binding. It is therefore probable that TR/RXR binds more tightly to nucleosomal DNA than histone H1 and that the two proteins either compete for a shared binding site that includes part of the dyad axis and/or the edge of the nucleosome, or that the association of TR/RXR alters the organization of the nucleosome in particular core–histone–DNA interactions such that stable binding of histone H1 can no longer be maintained. Protein–DNA crosslinking studies will help to resolve these issues.

Consistent with the continued access of TR/RXR to nucleosomal DNA in the presence of histone H1, the expression of histone H1 in oocytes has no functional consequences for TRβA promoter activity (Figure 5). These results differ from work indicating that inclusion of histone H1 invariably restricts transcription factor access to nucleosomal DNA (Juan *et al.*, 1994, 1997; Ura *et al.*, 1995, 1997). However, *in vivo* ablation experiments (Bouvet *et al.*, 1994) and genetic knock-outs of histone H1 (Shen and Gorovsky, 1996) indicate that histone H1 has a highly selective role in directing transcriptional repression. Future experiments will explore whether the architecture of the TRβA gene chromatin can be manipulated to create a dependence of transcriptional regulation on the incorporation of histone H1 into chromatin.

Materials and methods

Plasmids and DNA fragments

The pTRβA construct was generated by cloning a 1.9 kb *Eco*RI fragment containing 1.6 kb of TRβA promoter sequence and ~0.3 kb of CAT gene sequence from plasmid pCAT-WT (Ranjan *et al.*, 1994) into pBluescript II KS(–) (Stratagene, CA). The dsDNA of pTRβA was prepared using a Qiagen kit as described by the manufacturer. The ssDNA of pTRβA was prepared from phagemids induced with CVS M13 as described (Sambrook *et al.*, 1989). The TRE mutants were generated by PCR as described (Ranjan *et al.*, 1994). The DNA fragments used for nucleosome reconstitution were prepared with PCR using 20 nucleotide primers abutting either +159 or +363 relative to the start site of transcription (+1) or 20 nucleotide primers abutting either +159 or +536. The constructs in which the *X.borealis* somatic 5S rRNA gene nucleosome positioning sequences are fused to the TRE were made by PCR using one fixed primer at the 5′ end that includes an *Acc*I site and 5S sequences from –70 and one of two primers that extend either to +20 in the 5S gene (5S-A) or to +23 (5S-B). The amplified DNA fragment was inserted into the pTRβA construct between the *Acc*I–*Sty*I restriction endonuclease sites. These DNA fragments replaced the native sequence between +154 and +257. For the rotational positioning experiments, DNA fragments of 160 bp (5S-A) or 163 bp (5S-B) from +150 to +310 or +313 (relative to the start site of transcription at +1) were used for nucleosome reconstruction and TR/RXR binding experiments. To produce *Xenopus* RXRα mRNA for microinjection, the cDNA encoding *Xenopus* RXRα [the kind gift of Dr E.De Robertis (Blumberg *et al.*, 1992)] was cloned into pSP64 poly(A) vector. Briefly, cDNAs encoding *Xenopus* full-length RXRα was amplified by PCR with Vent polymerase (New England Biolabs) and then cloned into pSP64 poly(A) to generate pSP64(A)-xRXRα. The pSP64(A)-xTRβA, which contains cDNA encoding full-length TRβA receptor, was a kind gift from Drs A.Karamori and D.Brown at Carnegie Institute of Washington (Washington, DC).

Purification of nucleosome core particles, histone octamers and histone H1

Histone octamers were obtained from HeLa cells. HeLa nuclei and histone octamers were prepared as described by O'Neill *et al.* (1992). Nucleosome core particles (nucleosome monomer) were prepared from chicken erythrocyte nuclei (Tatchell and van Holde, 1977; Reeves and Nissen, 1993). Histone H1 was prepared from *Xenopus* erythrocytes by means of 5% perchloric acid extraction of nuclei and acetone precipitation (Hayes *et al.*, 1994).

Nucleosome reconstitution

Nucleosome cores were reconstituted onto radiolabeled DNA fragments either by exchange from chicken erythrocyte core particles or by dialysis from high salt with purified HeLa core histones (Tatchell and van Holde, 1977; Hansen *et al.*, 1991). In no circumstances did we detect differences in results due to the reconstitution methodology employed or the source of histones. In the histone exchange method, a 15-fold template mass excess of core particles was mixed with radiolabeled DNA in tubes followed by slow adjustment of NaCl concentration (to 1 M). Tubes were incubated at 37°C for 15 min. Samples were transferred to a dialysis bag (with a molecular size limit of 6–8 kDa) and dialyzed against 1.0 M NaCl, 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 0.1 mM phenylmethylsulfonylfluoride (PMSF) and 1 mM 2-mercaptoethanol for 4 h at 4°C, then dialyzed in 0.75 M NaCl buffer for 4 h, followed by a final dialysis against 10 mM Tris–HCl, pH 7.5, 0.1 mM EDTA and 1 mM 2-mercaptoethanol overnight.

For the salt dialysis method using purified histone octamers, radiolabeled DNA (500 ng) and unlabeled DNA (4.5 μg) were mixed with histone octamers in 2.0 M NaCl. The final DNA concentration was 0.1 mg/ml, and the histone octamer concentration was 1.0 mol of octamer/mol of DNA repeat. Samples were then dialyzed at 4°C against 10 mM Tris–HCl, pH 7.5, 1 mM EDTA, 0.1 mM PMSF, 1 mM 2-mercaptoethanol and NaCl as follows: 2.0 M NaCl, 1 h; 1.5 M NaCl, 4 h; 1 M NaCl, 4 h; 0.75 M NaCl, 4 h. The final dialysis was overnight into 10 mM Tris–HCl, pH 7.5, 1 mM EDTA and 1 mM 2-mercaptoethanol at 4°C.

After reconstitution, the oligonucleosome cores were loaded on 5–20% sucrose gradients containing 10 mM Tris–HCl, pH 7.5, 1 mM EDTA and 0.1 mM PMSF, and then centrifuged for 16 h at 35 000 r.p.m. at 4°C in a Beckman SW41 rotor. Fractions were collected and analyzed by nucleoprotein agarose (0.7%) gel electrophoresis in 0.5× TBE (1× TBE is 90 mM Tris base/90 mM boric acid/2.5 mM EDTA) (Figure 2A). Fractions containing mono-, di- and trinucleosomes were pooled separately, concentrated to ~2.5 μg/ml using microcon-30 (Amicon) and dialyzed against 10 mM Tris–HCl, pH 7.5, 0.1 mM EDTA, 1 mM 2- mercaptoethanol overnight at 4°C. Samples were stored on ice until used, at which time their integrity was monitored by electrophoresis (Godde and Wolffe, 1995). Chicken erythrocyte oligonucleosomes (chromatin lengths of 1–30 nucleosomes) were prepared, after removal of linker histones (Lee *et al.*, 1993), and used as a control for isolation of the native dinucleosome complex.

H1 binding experiments

A total of 100 ng (DNA content) of reconstituted nucleosome cores (0.6 pmol) were incubated with various amounts of histone H1 in 10 μl of binding buffer [10 mM Tris–HCl, pH 8.0/50 mM NaCl/0.1 mM EDTA/5% (v/v) glycerol] at room temperature for 15–30 min (Hayes and Wolffe, 1993). Samples were loaded directly onto running 0.7% agarose gel in 0.5× TBE. After electrophoresis, the gels were dried and autoradiographed.

Micrococcal nuclease mapping of translational positioning

Monosomes and dinucleosomes (80 ng DNA) (molar ratio of histone to DNA = 1) were digested with 0.075–0.6 U of micrococcal nuclease (Pharmacia) for 5 min at 22°C. Incubation with H1 was as described above. Ca^{2+} was adjusted to 0.5 mM concomitantly with addition of micrococcal nuclease. Digestions were terminated with addition of EDTA (5 mM), SDS (0.25%, wt/v), and proteinase K (Gibco-BRL) (1 mg/ml). The DNA was recovered and 5′-end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase, and the end-labeled DNA fragments were separated by electrophoresis in non-denaturing a 6% polyacrylamide gels. DNA fragments of nucleosome core and chromatosome products were recovered and digested with restriction endonucleases to determine micrococcal nuclease cleavage sites (Hayes and Wolffe, 1993)

DNase I and hydroxyl radical footprinting of rotational positioning

Reconstitutes with or without histone H1 were treated with either DNase I or hydroxyl radicals before resolving nucleoprotein complexes on preparative 0.7% agarose gels (Hayes and Wolffe, 1992). Samples contained labeled mononucleosomes or dinucleosomes (60 ng DNA) and chicken erythrocyte core particles (~1 μg), and were incubated with or without 200 ng histone H1 (1 molecule/nucleosome core) as described above. Mg^{2+} was adjusted to 4 mM concomitantly with addition of

DNase I. Naked DNase was digested with 12 ng of DNase I (Gibco-BRL), nucleosome cores without H1 were digested with 30–60 ng enzyme, and nucleosome cores with H1 were digested with 480–960 ng enzyme. DNase I reactions were carried out at room temperature for 1 min and terminated by addition of EDTA (5 mM). Glycerol (5%, v/v) was added to the sample and the entire reaction volume was transferred directly into a preparative gel. The hydroxyl radical reaction were carried out as described by Hayes *et al.* (1990). Free radical reactions were quenched with the addition of glycerol to a concentration of 5%, and the entire volume was applied to a gel, as described above. After electrophoresis, bound or unbound H1 nucleosome complexes were excised from gel. DNA from these complexes was isolated and analyzed by denaturing polyacrylamide (6%) gel electrophoresis. Specific DNA makers were produced by Maxam and Gilbert cleavage at G residues.

Microinjection of Xenopus oocytes

The preparation of *Xenopus* stage VI oocytes and the microinjection procedure were essentially as described (Almouzni and Wolffe, 1993). The pTRβA DNA was injected (23 nl/oocyte) either as ssDNA (1.15 ng/oocyte) or dsDNA (2.3 ng/oocyte) into the nuclei (GV) of the oocytes, and the indicated amounts of mRNAs for *Xenopus* TRβA and RXRα were injected (27 nl/oocyte) into the oocyte cytoplasm. The mRNAs were injected 6 h before the injection of DNA. For transcription analysis, ~20 oocytes were injected for each sample to minimize the variations among oocytes and injections. The injected oocytes were incubated at 18°C overnight in MBSH buffer (Almouzni and Wolffe, 1993) supplemented with antibiotics (50 U/ml ampicillin and streptomycin) and then collected for transcription analysis or DNA analysis as described below.

Transcription analysis and DNA analysis

Transcription analysis by primer extension and recovery of DNA from the injected oocytes were performed essentially as described (Almouzni and Wolffe, 1993). Briefly, ~20 injected oocytes were collected for each sample, rinsed with 400 µl of MBSH buffer, and then homogenized in 300 µl of 0.25 M Tris, pH 8.0. Half of the sample was used to isolate RNA using RNAzol™ reagent, and the remaining half was used for DNA recovery. To isolate RNA, 500 µl of RNAzol™ reagent was added to the sample, vortexed, and then incubated on ice for 15 min before centrifugation. The clean supernatant was transferred to a new tube and extracted once with equal volume of phenol/chloroform. The RNA was then precipitated with 0.7 vol of isopropanol, rinsed with 70% ethanol, and dissolved in DEPC-treated water. For primer extension analysis, RNA from one or two oocyte equivalents was annealed with the end-labeled primer I 5′-ATCCTTATAAACGGTGAGTAGTGATGTCAT-3′ in 10 µl of 0.4 M KCl at 65°C for 10 min, 55°C for 25 min and 42°C for 5 min. 30 µl of reverse transcription mixture [67 mM Tris–HCl at pH 8.3, 8 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 1 mM dNTP mix, 1 unit of RNasin, and 10 units of Superscript II™] was then added. The reaction was incubated at 42°C for 1 h and then stopped by ethanol precipitation. The products were displayed on a 6% sequencing gel and visualized by autoradiography.

For DNA recovery, the remaining half of the sample was mixed with equal volume of 20 mM EDTA, 0.2% SDS and then treated with protease K (100 µg/ml) at 55°C for 2–3 h. After two phenol/chloroform extractions, the sample was precipitated with isopropanol and then resuspended in 100 µl of TE buffer. DNA was then treated with RNase A (100 µg/ml) at 37°C for 1 h followed by phenol/chloroform extraction and ethanol precipitation. DNA was quantitatively analyzed either by Southern blot as described (Sambrook *et al.*, 1989). Blots were probed with ssDNA of pTRβA labeled with a random prime labeling kit (Amersham) as described by the manufacturer.

Gel retardation assay of the binding of TR/RXR heterodimer to the TRE reconstituted into nucleosome in vitro

To examine the *in vitro* reconstitution of the TRE of the TRβA promoter into a nucleosome, PCR products as indicated above were gel purified. The reconstitution was accomplished with the octamer exchange method as described (Hayes and Wolffe, 1992) using the histone octamers purified from chicken erythrocytes. The gel retardation assay was performed essentially as described (Ranjan *et al.*, 1994) except the oocyte produced TR/RXR heterodimer was used. Briefly, the oocytes were injected with TR/RXR mRNAs (2.7 ng/oocyte each). After overnight incubation, the injected and uninjected oocytes were rinsed with TE buffer once and then homogenized in 20 µl/oocyte of buffer E (10 mM Tris–HCl, pH 7.6, 60 mM KCl, 0.1 mM EDTA, 5 mM DTT, 1 mM PMSF and 8% glycerol). The extracts used for binding assays were obtained by microcentrifuging the crude extracts at 10 000 *g* at 4°C for 20 min to remove yolk proteins and lipids. The mixtures were loaded on a 4% native polyacrylamide gel containing 3% glycerol in 0.5× TBE prerun at room temperature for 1 h (150 V) and run for a further 2 h. As controls, binding reactions were done in 25 µl volume with free probe alone or free probe plus 2 µl of TR/RXR oocyte extract.

Supercoiling assay for chromatin disruption

The supercoiling assay using chloroquine agarose gel was performed essentially as described (Clark and Wolffe, 1991). To analyze DNA topology, 10 µl of each DNA sample was loaded onto a 1–1.2% agarose gel in 1× TPE (40 mM Tris, 30 mM NaH_2PO_4, 10 mM EDTA) containing 90 µg/ml of freshly prepared chloroquine in both gel and running buffer. Under these gel conditions, the minichromosomes with nucleosome density from 180 to 270 bp/nucleosome can be resolved optimally into isomers with positive supercoiling due to the binding of chloroquine to DNA, dependent on the differences in negative supercoiling which resulted from the difference in nucleosome density. The gels were run in the dark overnight at ~3.5 V/cm. The gels were then washed with distilled water several times to remove chloroquine and processed for DNA transfer to Nytran Plus membrane as described (Sambrook *et al.*, 1989). The DNA was probed with a random-primed labeled DNA fragments as described above.

DNase I-hypersensitive site analysis

Briefly, groups of 25–30 injected oocytes were collected after overnight incubation and homogenized in 400 µl of MNase buffer (10 mM HEPES, pH 8.0, 50 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.1% NP-40, 5% glycerol). The extract was divided into four fractions (100 µl each) and digested with 80, 40, 20 and 10 U of DNase I (BRL) respectively at room temperature for 1 min. The reactions were stopped with addition of 200 ml of 1% SDS and 20 mM EDTA. After the purification procedure as described for the MNase assay, each DNA sample was resuspended in 100 ml of TE buffer and treated again with RNase A (100 µg/ml) for 1 h, followed by phenol/chloroform extraction and isopropanol precipitation to further clean the DNA samples. The DNA samples were then resuspended in 10 ml of 1× *Eco*RI buffer (BRL) and digested with 15 U of *Eco*RI (BRL) for 2 h. The digested DNA samples were resolved by a 1.2% agarose gel, blotted to Nytran Plus membrane and probed with a random primer-labeled CAT fragment (+506 to +584). The naked DNA controls were treated with 200-fold less DNase I than used for chromatin samples.

Micrococcal nuclease mapping of nucleosome positions by indirect end-labeling

The MNase assay of chromatin structure was performed as described previously (Wong *et al.*, 1995). Briefly, groups of 20–25 injected oocytes were collected after overnight incubation and homogenized in 300 ml of MNase buffer (10 mM HEPES, pH 8.0, 50 mM KCl, 5 mM $MgCl_2$, 3 mM $CaCl_2$, 1 mM DTT, 0.1% NP-40, 5% glycerol). The extract was either divided into four fractions (60 µl each) and digested with 10, 5, 2.5 and 1.25 U/ml of MNase (Worthington) respectively at room temperature for 20 min. MNase digestions were stopped by addition of 200 ml of 20 mM EGTA, 1% SDS. The reactions were treated with RNase A (100 µg/ml) for 2–3 at 55°C. After addition of 30 µl of 3 M NaOAC, pH 5.4, the reactions were phenol/chloroform extracted twice, precipitated with 0.7 vol. of isopropanol, and rinsed with cold 70% ethanol. DNAs were digested with *Eco*RI and resolved by 1.5% agarose gel, blotted to nylon membrane and probed with a radiolabeled DNA fragment abutting the *Eco*RI site at the 3′ end of the CAT gene from +506 to +584.

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